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ORIGINAL ARTICLE

Laboratory diagnosis of vaccine-associated measles in Zhejiang Province, China

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KEYWORDS
allelic discrimination;
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Abstract *Background/Purpose:* Along with the improving vaccine coverage, suspected vaccine-associated measles has been reported in Zhejiang Province, China. In order to maintain the accuracy of the measles surveillance system, it is critical to discriminate between measles vaccine and wild-type virus.
Methods: Eight suspected cases of vaccine-associated measles were reported in Zhejiang Province during 2011 and 2014. Sera collected within 4 days and throat swabs collected within 6 days after rash onset were tested with immunoglobulin M and measles virus (MeV) RNA to confirm MeV infection. In order to further identify the vaccine-associated cases, throat swabs with positive MeV RNA were tested using an allelic discrimination real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay developed in this study, RT-PCR-restriction fragment length polymorphism (RFLP) recommended by the National Measles Laboratory, and RT-PCR followed by sequencing and genotyping.
Results: Combining anti-measles immunoglobulin M and RNA testing, eight cases were confirmed as MeV infection. Of the eight, two were identified as vaccine-associated cases by the allelic discrimination rRT-PCR assay, and one was identified by RT-PCR-RFLP. Subsequent sequencing and genotyping confirmed that the sequences of the two cases were identical to that of the Chinese vaccine strain. The developed allelic discrimination rRT-PCR was 10 times more sensitive than the RT-PCR-RFLP assay when RNA standards generated from three genotypes of MeV were tested.

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Conclusion: Vaccine-associated measles has been identified in Zhejiang. The developed allelic discrimination rRT-PCR assay is rapid and sensitive, which will facilitate the surveillance for vaccine-associated measles.

Introduction

Measles is a highly contagious disease, which resulted in millions of deaths in the pre-vaccine era. The morbidity and mortality of measles have declined considerably since the 1960s, when the attenuated or killed measles vaccine was developed and used successfully throughout the world.[1,2] After decades of improved immunization coverage and decreasing incidence, the elimination of measles became the goal of the World Health Organization (WHO) in its four regions by 2015.[3] To achieve this goal in China, WHO has recommended a measles mass immunization campaign (MMIC) in addition to two doses of routine vaccination.

Measles-containing vaccines, the most effective means to control and interrupt measles infection at present,[4] have been used globally for ~50 years. Although the vaccines have been proved to be safe and effective, previous studies have found that the live attenuated vaccine may cause measles-like symptoms, such as moderate fever, rash, and conjunctivitis, in 5—15% of recipients.[5,6] With the apparent drop in the incidence of wild-type measles, the epidemiology and clinical pattern of the disease have changed, and vaccine-associated measles has been reported in many countries.[7—10] In order to ensure the accuracy of the measles surveillance system during the period of measles elimination, vaccine-associated measles should be distinguished from wild-type measles virus (MeV) infection when cases are reported.

Differentiation of vaccine-associated measles based on clinical symptoms or laboratory methods routinely used in MeV surveillance, such as immunoglobulin M (IgM) testing, virus isolation, and RNA amplification, is unworkable or unreliable. Sequencing combined with genotyping has always been the most accessible way to identify vaccine-associated cases[11]; however, this method is time consuming, and not every clinical specimen contains sufficient amount of viral RNA to perform sequencing analysis.[12] A reverse transcriptase polymerase chain reaction-restriction fragment length polymorphism (RT-PCR-RFLP) assay has been developed and recommended for discriminating between measles vaccine and wild-type strains by the Chinese National Measles Laboratory.[13] The process of the RT-PCR-RFLP is divided into RT-PCR plus enzyme digestion and gel electrophoresis, which are not only labor intensive but can easily cause crossover contamination as well. A loop-mediated isothermal amplification assay has also been developed for differentiating vaccine from wild-type MeVs in Japan.[12] This assay is simpler than RT-PCR-RFLP, but its sensitivity for detecting wild-type MeV strains is ~10 times lower than it is for detecting vaccine-type strains.[12]

In this paper, we describe the differentiation process of eight suspected vaccine-associated measles monitored in Zhejiang Province, China. We have developed an allelic discrimination real-time RT-PCR (rRT-PCR) assay and applied it to discriminate between measles vaccine and wild-type strains. Our research provides data for the laboratory diagnosis of vaccine-associated measles case and contributes to the accuracy of the measles case surveillance system.

Methods

Suspected vaccine-associated measles

Eight suspected vaccine-associated measles were reported in Zhejiang Province, China during 2011 and 2014. All the suspected cases were vaccinated with live attenuated measles and rubella combined vaccine 4—11 days prior to the onset of rash. Patients presented with symptoms of fever and rash, which are consistent with measles clinically (Table 1). Cases 1 and 7 were reported to present with typical Koplik's spots. Cases 2—4 and 6 presented with symptoms of cough, conjunctivitis, and coryza (3C). Case 1 was also reported to have enlarged lymph nodes. Cases 1, 2, and 6 had been hospitalized prior to onset of illness (Table 1).

Specimens

Clinical specimens of eight suspected vaccine-associated cases were used in this study. Serum samples collected within 4 days after rash onset were tested by enzyme-linked immunosorbent assay kit (Virion\Serion, Würzburg, Germany). Throat swab specimens collected within 6 days after rash onset were analyzed by a multiplex rRT-PCR for MeV and rubella virus (RV) routinely used in Zhejiang Provincial Center for Disease Control and Prevention (CDC),[14] as well as used for virus isolation.[15] Afterwards, throat swabs with positive MeV RNA were tested using the allelic discrimination rRT-PCR developed in this study, RT-PCR-RFLP recommended by the National Measles Laboratory,[13] and RT-PCR followed by sequencing and genotyping.[16]

RNA extraction

Total RNA was extracted from clinical specimens or cell culture supernatants using the High Pure Viral Nucleic Acid Kit (Roche, Welwyn Garden City, UK). Briefly, 200 μL of clinical specimen or cell culture supernatant was added to a lysis buffer containing poly (A) and proteinase K and

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Table 1 Clinical information for eight suspected vaccine-associated measles

Case No.	Date of birth	Date of vaccination	Date of fever	Date of rash onset	Clinical symptoms					Ever been hospitalized
					Koplik's spots	Cough	Coryza	Conjunctivitis	Enlargement of lymph nodes	
1	28 Jul 2010	18 Apr 2011	27 Apr 2011	28 Apr 2011	√				√	Yes
2	1 Jan 2012	15 Mar 2013	22 Mar 2013	25 Mar 2013		√	√	√		Yes
3	24 Jul 2012	26 Mar 2013	2 Apr 2013	4 Apr 2013		√				No
4	9 Dec 1986	4 Apr 2013	8 Apr 2013	10 Apr 2013				√		No
5	3 Sep 2012	3 May 2013	11 May 2013	12 May 2013						No
6	10 Jul 2012	18 Mar 2013	19 Mar 2013	22 Mar 2013		√	√	√		Yes
7	18 Jun 2013	20 Feb 2014	28 Feb 2014	2 Mar 2014	√					No
8	13 Jun 2013	13 Feb 2014	22 Feb 2014	24 Feb 2014						No

incubated at 72°C for 10 minutes. The sample was run through the High Pure Spin Filter column and the RNA was bound to the glass fiber fleece. The contaminants, including salts, protein, and cellular contaminants, were removed through several wash and spin steps. The purified RNA was eluted into 50 μL Elution Buffer and then stored at −80°C.

Allelic discrimination rRT-PCR assay

Whole-genome sequences of 16 MeVs, including seven vaccine strains (genotype A) and nine wild-type strains (genotypes H1a, H1b, B3, D3, D5, D6, D7, D8, and G3), were downloaded from GenBank. Multiple sequence alignments were performed using BioEdit software (Figure 1). The allelic discrimination site was searched through the whole genome. Primer pair and TaqMan-minor groove binder (MGB) probes containing the allelic discrimination site were designed using PrimerExpress 3.0 software (Applied Biosystems, Foster City, CA, USA). Sequences and positions of the primer pair and TaqMan-MGB probes are shown in Table 2. The probe for detecting wild-type MeV (wild-type probe: 5′-FAM-CTTCCAGCAGGCG-NFQ-MGB) was labeled with FAM (6-carboxy-fluorescein) at the 5′ end, while the probe for detecting measles-vaccine strain (Vaccine probe: 5′-VIC-CATGCTTCCAACAGG-NFQ-MGB) was labeled with VIC fluorescence. MGB was labeled at the 3′ end of the two probes.

The allelic discrimination rRT-PCR was performed using One Step PrimeScript RT-PCR (Perfect Real-time) kit (TaKaRa Biotechnology Co., Kusatsu, Japan). Each 25-μL reaction mixture contained 12.5 μL 2× One Step RT-PCR Buffer, 0.5 μL TaKaRa Ex Hot start (HS) Taq, 0.5 μL PrimeScript RT Enzyme Mix, 0.5 μL each primer, 0.25 μL each probe, 5 μL deionized distilled water, and 5 μL RNA template. The final concentration of each primer and probe was 0.4μM and 0.2μM, respectively. The optimal amplification conditions are shown in Table 2. FAM and VIC fluorescence signals were acquired at the end of each annealing step.

Preparation of MeV RNA standards

Three MeV strains were used to prepare RNA standards. Measles vaccine was produced by Shanghai Institute of Biological Products, Shanghai, China, of which genotype A

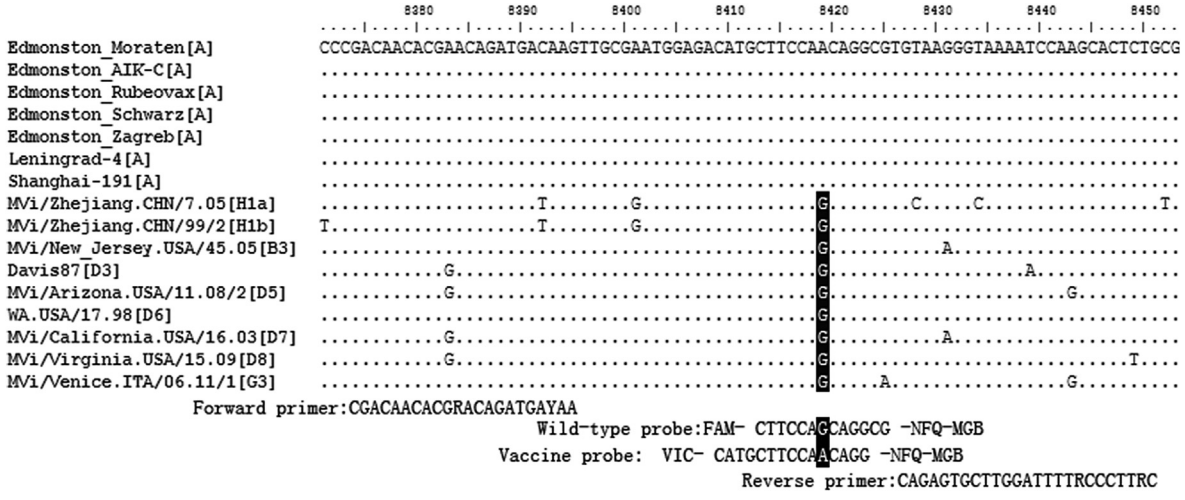

Figure 1. Primer pair and probes designed for the allelic discrimination real-time reverse transcriptase polymerase chain reaction assay. The upper part shows sequence alignments between seven vaccine strains and nine wild-type strains. The lower part shows sequences of the primer pair, vaccine probe and wild-type probe. The highlighted location indicates the nucleotide that is different between vaccine and wild-type strains. The vaccine probe and wild-type probe is labeled with VIC and FAM (6-carboxy-fluorescein) fluorescence, respectively, at the 5′ end.

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Table 2 Details of the allelic discrimination rRT-PCR assay

Items	Allelic discrimination rRT-PCR		
Primer pair (20 pmol/μL)	Forward	5′-CGACAACACGRACAGATGAYAA-3′ (nt 8373—8394)	
	Reverse	5′-CAGAGTGCTTGGATTTTRCCCTTRC-3′ (nt 8451—8427)	
Probes (20 pmol/μL)	Wild type	5′-FAM- CTTCCAGCAGGCG-NFQ-MGB-3′ (nt 8413—8425)	
	Vaccine	5′-VIC-CATGCTTCCAACAGG-NFQ-MGB-3′ (nt 8409—8423)	
Program	42°C	15 min	
	95°C	2 min	
	94°C	10 sec	
	60°C	35 sec	40 cycles
	Data collection		
Sensitivity			
RNA standard of genotype A virus	10 copies/μL		
RNA standard of genotype H1 virus	10 copies/μL		
RNA standard of genotype D4 virus	10 copies/μL		
Time for process	~1.5 h		
Application	Discrimination between vaccine & wild-type measles virus		

rRT-PCR = real-time polymerase chain reaction.

strain Shanghai-191 (GenBank accession No. FJ416067) was incorporated. Genotype H1 strain MVi/Zhejiang.CHN/12.09/1 was isolated from an acute measles case in Zhejiang CDC. Genotype D4 strain was donated by the National Measles Laboratory.

Three genotypes of RNA standard were generated as previous described.[17] H gene fragment [nucleotides (nt) 7178—9224] was amplified using RNA template of three MeV strains with primers containing T7 promoter sequence and were *in vitro* transcribed with T7 RNA polymerase (TaKaRa Biotechnology Co.). The sequence of the forward primer was 5′-ACTCGTTAATACGACTCACTATAGGGAG CCAGCATCAATCCCACCTGAA-3′ (T7 promoter sequence were highlighted in bold) (nt 7178—7198) and the reverse primer was 5′-CCACTTGGACCCTACGTT-3′ (nt 9224—9207). The synthetic RNA transcripts were purified, qualified and then 10-fold diluted, ranging from 10^6 copies/μL to 10^0 copies/μL.

Specificity and sensitivity of the allelic discrimination rRT-PCR

Specificity of the allelic discrimination rRT-PCR assay was determined using RNA extracted from cell culture supernatant of three genotypes of MeV, RV, mumps virus, and influenza virus subtype A, as well as the clinical specimens from patients infected with enterovirus 71 and coxsackievirus A16. Sensitivity was assessed through performing the allelic discrimination rRT-PCR in parallel with the RT-PCR-RFLP on serial diluted (10^6—10^0 copies/μL) RNA standards generated from three genotypes of MeV. Throat swabs from eight suspected vaccine-associated cases were also used to validate the developed assay.

RT-PCR-RFLP

We used the RT-PCR-RFLP assay recommended for discriminating between MeV vaccine and wild-type strains in China.[13] A fragment located within nt 7516—7953 on the MeV H gene was amplified by RT-PCR. The 438 nt RT-PCR product contained a restriction enzyme cutting site at nt 7666—7671. Measles vaccine strain (genotype A) can be cut into two fragments (287 nt and 151 nt length bands) after enzyme digestion; however, none of the wild-type strains can be cut by enzyme, and only one band can be observed in gel electrophoresis.

RT-PCR followed by sequencing and genotyping

The 450 nt sequence at the C terminus of the N gene was amplified by RT-PCR using PrimeScript One Step RT-PCR Kit (TaKaRa Biotechnology Co.) and primer pair MeV214 and MeV216 that were routinely used to perform genotyping of MeV.[16] Five microliters of RNA was used as a template for RT-PCR. The first round of nested RT-PCR was generated by primer pair MeV214 and MeV216.[16] The primer pair for the second round was originated from MeV214 and MeV216 with slight modifications. The sequence of the forward primer was 5′-GGAGCTATGCCATGGGAGTAG-3′ (nt 1105—1125) and the reverse primer was 5′-AACAATGATGGAGGGTAGGCG-3′ (nt 1736—1716). RT-PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by Sangon Biotech (Shanghai, China). The phylogenetic tree was drawn based on the 450 nt sequences of 23 reference strains,[18] and six sequences generated in this study (Table 3), as well as Chinese vaccine strain Shanghai-191 (GenBank accession No. FJ416067), using the neighboring-joining and the Kimura two-parameter model of MEGA version 5.0 software (http://www.megasoftware.net).[19]

Results

Confirmation of MeV infection

For case confirmation, anti-measles IgM testing, MeV and RV RNA amplification, and virus isolation were performed. Of the eight cases, six were positive for anti-measles IgM, but

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Table 3 Laboratory test results for eight suspected cases of vaccine-associated measles

Case No.	Date sample collection		IgM	Multiplex rRT-PCR	Virus isolation	Developed rRT-PCR		RT-PCR-RFLP		RT-PCR	Sequence name (genotype)
	Sera	Throat swab				Results	Channel	Results	No. band		
1	29 Apr 2011	30 Apr 2011	Pos	Pos/MeV[a]	Neg	Pos	VIC	Pos	Two	Pos	MVs/Zhejiang.CHN/17.11 (A)
2	25 Mar 2013	28 Mar 2013	Pos	Pos/MeV	Pos	Pos	FAM	Pos	One	Pos	MVi/Zhejiang.CHN/13.13/12 (H1)
3	4 Apr 2013	4 Apr 2013	Pos	Pos/MeV	Pos	Pos	FAM	Pos	One	Pos	MVi/Zhejiang.CHN/14.13/14 (H1)
4	11 Apr 2013	11 Apr 2013	Neg	Pos/MeV	Pos	Pos	FAM	Pos	One	Pos	MVi/Zhejiang.CHN/15.13/1 (H1)
5	14 May 2013	15 May 2013	Pos	Pos/MeV	Neg	Pos	FAM	Neg		Neg	
6	25 Mar 2013	28 Mar 2013	Pos	Pos/MeV	Pos	Pos	FAM	Pos	One	Pos	MVi/Zhejiang.CHN/12.13/8 (H1)
7	6 Mar 2014	6 Mar 2014	Pos	Pos/MeV	Neg	Pos	FAM	Neg		Neg	
8	26 Feb 2014	25 Feb 2014	Neg	Pos/MeV	Neg	Pos	VIC	Neg	Neg	Pos[b]	MVs/Zhejiang.CHN/12.14 (A)

[a] Pos/MeV means positive for MeV RNA.
[b] Sequence for genotyping was obtained by nested RT-PCR.
FAM = 6-carboxy-fluorescein; IgM = immunoglobulin M; MeV = measles virus; Neg = negative; POS = positive; rRT-PCR = real-time polymerase chain reaction; VIC = .

Cases 4 and 8 whose serum specimens were collected within 2 days after rash onset gave negative results (Table 3). All eight cases were positive for MeV RNA but negative for RV RNA (Table 3). Combining the results of anti-measles IgM and MeV RNA, the eight cases were all confirmed as MeV infection. In addition, four MeV strains were isolated from Cases 2–4 and 6 (Table 3).

Evaluation of the allelic discrimination rRT-PCR assay

An allelic discrimination rRT-PCR assay was developed for differentiating between MeV vaccine and wild-type strains. Based on the result of multiple sequence alignments on the MeV genome (Figure 1), site nt 8419 located within the H gene was selected as an allelic discrimination site. MeV vaccine strains contain nucleotide A, while wild-type strains contain nucleotide G (Figure 1) on the site. Two TaqMan-MGB probes used in the assay were designed to hold the allelic discrimination site (nt 8419) and were labeled with different fluorescence (VIC and FAM). MeV vaccine and wild-type strains can be differentiated based on fluorescence signals acquired after amplification.

Specificity evaluation of the allelic discrimination rRT-PCR showed that the assay has no cross-reactivity against other respiratory viruses, except for three genotypes of MeV. Moreover, positive result on the VIC channel was only generated by genotype A MeV, while positive results on the FAM channel were detected from genotype H1 and D4 MeVs.

Sensitivity of the allelic discrimination rRT-PCR was assessed and compared with the RT-PCR-RFLP assay. When RNA standards were tested, detection limits of the allelic discrimination rRT-PCR assay were 10 copies/μL for three genotypes of MeV (Figure 2 and Table 2), while the lowest concentration RT-PCR-RFLP assay could detect was 100 copies/μL. Through enzyme digestion, two specific bands with 287 nt and 151 nt in length were observed in the gel picture of genotype A RNA standard (Figure 2B), but only one band of 438 nt in length was observed in the picture of genotype H1 or D4 RNA standards, respectively (Figure 2D and F). In addition, the allelic discrimination rRT-PCR was faster than RT-PCR-RFLP, as the whole process only needed ~1.5 hours.

Identification and confirmation of vaccine-associated cases

In order to discriminate whether or not the eight cases were caused by measles vaccine, throat swabs were tested using the allelic discrimination rRT-PCR in parallel with RT-PCR-RFLP assay. All the cases tested positive for MeV RNA using allelic discrimination rRT-PCR, two of which (Cases 1 and 8) acquired positive results from channel VIC and were identified as vaccine-associated measles, while the other six were tested as positive in channel FAM and identified as wild-type MeV infection (Table 3). Five (Cases 1–4 and 6) of the eight cases were detected as positive by the RT-PCR-RFLP assay, among which only Case 1 was identified as a vaccine-associated case, as there were two bands observed in the electrophoretogram (Table 3).

RT-PCR followed by sequencing and genotyping was used to confirm the results of allelic discrimination rRT-PCR. Six of the eight tested positive by RT-PCR, of which, two (MVs/Zhejiang.CHN/17.11 and MVs/Zhejiang.CHN/12.14) were directly generated from clinical specimens of Cases 1 and 8, and another four were generated from cell culture supernatants. The sequence of Case 8 was generated by nested RT-PCR, since there was a weak band after routine RT-PCR amplification.

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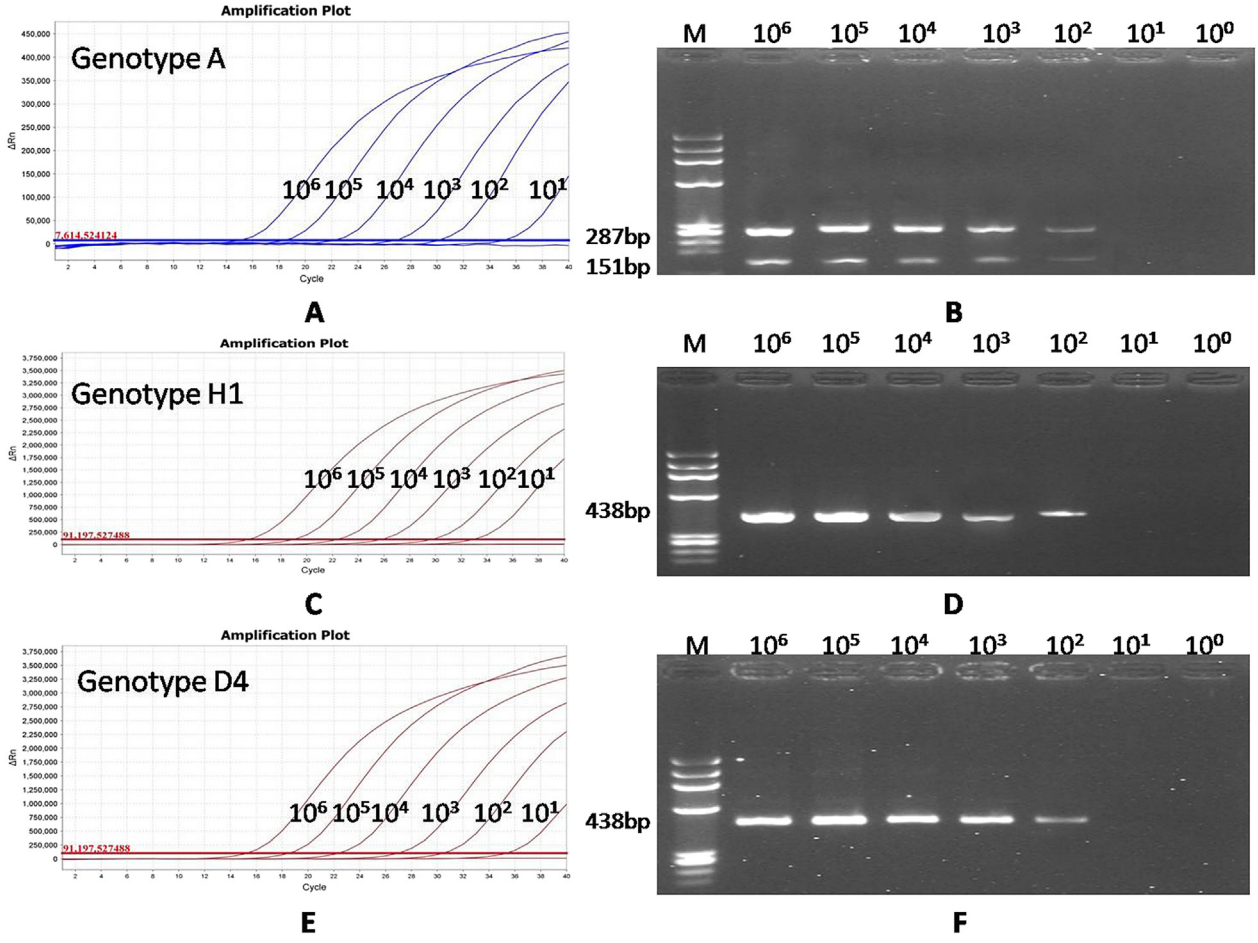

Figure 2. Sensitivity evaluation of the allelic discrimination rRT-PCR using RNA standards generated from three genotypes of measles virus. A and B indicate the detection limits of the allelic discrimination rRT-PCR and RT-PCR-RFLP for genotype A RNA standard. C and D show the detection limits of the two assays for genotype H1 RNA standard. E and F show the sensitivity for genotype D4 RNA standard. The genotype of each RNA standard was labeled on the top left of A, C and E. The size of the DNA marker was labeled on the left of the electrophoretogram. The dilutions of the RNA standard ranged from 10^6 copies/μL to 10^0 copies/μL. M = DNA marker; RFLP = restriction fragment length polymorphism; rRT-PCR = real-time reverse transcriptase polymerase chain reaction.

The phylogenetic tree (Figure 3) showed that MVs/Zhejiang.CHN/17.11 and MVs/Zhejiang.CHN/12.14 were located in the same branch with Edmonston-wt. USA/54, the reference strain of genotype A MeV, and were identical to the Chinese vaccine strain Shanghai-191 (GenBank accession No. FJ416067). Hence, these two were confirmed as vaccine-associated measles. The other four sequences generated from Cases 2—4 and 6 had the nearest distances to reference strain Hunan.CHN/93/7, and were identified as genotype H1 strains. Three of these four sequences (Cases 3, 4, and, 6) were identical and had seven nucleotides different from the MVi/Zhejiang.CHN/13.13/12 strain, which was generated from Case 2.

Discussion

With the acceleration of measles control and elimination in recent years, atypical and vaccine-associated measles have been monitored worldwide.[7—10] Many cases suspected to correlate with vaccine administration have been reported in Zhejiang Province, China, since a nationwide MMIC carried out in 2010 when >2 million children aged from 8 months to 4 years were immunized in Zhejiang Province with measles-containing vaccine.[20]

In this study, we focused on eight suspected cases of vaccine-associated measles that occurred in Shaoxing City of Zhejiang Province during 2011 and 2014. On arrival at the hospital, all of the patients presented with symptoms of fever and red maculopapules. They were clinically diagnosed as measles and reported to the local CDC. It was subsequently discovered that they had been immunized with measles and rubella combined vaccine 4—11 days prior to rash onset. Based on the first definition of vaccine-associated measles by the WHO,[21—23] hospital staff doubted whether the cases were caused by the measles vaccine. In order to determine if there was a connection, serum and throat swab specimens were collected and used to perform laboratory diagnosis.

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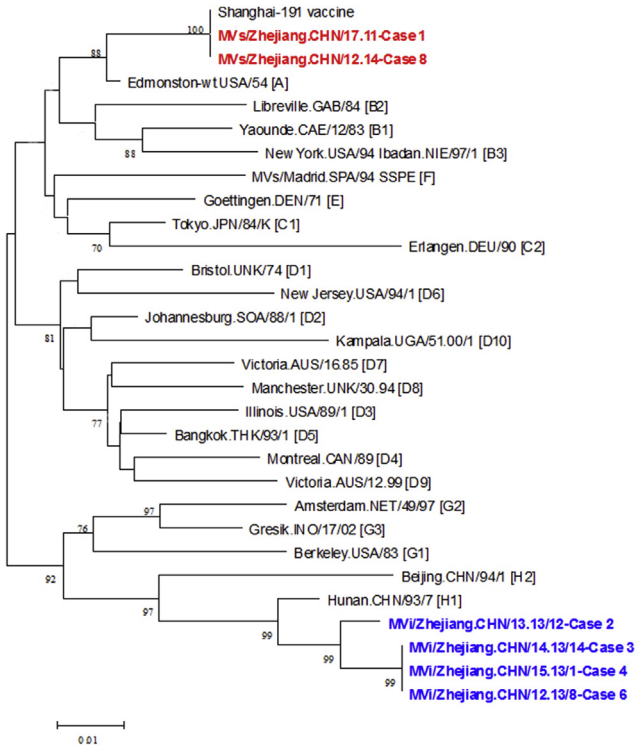

Figure 3. Phylogeny analysis of the suspected vaccine-associated cases. This phylogenetic tree was constructed based on the 450 nucleotide sequence of N gene generated from six suspected vaccine-associated measles and the reference strain for each genotype using the neighboring-joining and Kimura two-parameter model of the MEGA version 5.0 software. The reliability of the constructed tree was estimated by bootstrap analysis with 1000 replicates. Bootstrap values $>$ 70 were labeled above the nodes. The sequences of 23 reference strains were downloaded from GenBank. The genotype of the reference strain was marked within square brackets at the end of the name. The two sequences identified as genotype A by allelic discrimination real-time reverse transcriptase polymerase chain reaction were highlighted in red and the four genotype H1 sequences were highlighted in blue.

The clinical specimens were first tested for anti-measles IgM, and six cases were in accordance with the second definition of vaccine-associated measles, that is, blood samples collected 8—56 days after vaccination should be positive for anti-measles IgM.[21—23] However, we could not exclude the two IgM-negative cases (Cases 4 and 8), since up to 30% of tests for measles-specific IgM may give false-negative results in the first 3 days after rash onset.[21,24] Indeed, the two cases with negative IgM were subsequently confirmed as measles as well through MeV RNA amplification.

It is difficult to distinguish vaccine-associated measles from the wild-type infection using routine methods. Therefore, we developed an allelic discrimination rRT-PCR assay in this study, in order to improve the accuracy of differential diagnosis for these eight cases. Using vaccine-specific and wild-type-specific probes containing the allelic discrimination site (nt 8419), the measles vaccine strain could be easily distinguished from 22 genotypes of wild-type MeV, based on VIC fluorescent signals. Of the eight cases, two were identified as vaccine-associated measles by the new rRT-PCR method. One of these cases (Case 1) was also identified by the recommended RT-PCR-RFLP assay. However, Case 8 was tested as negative by RT-PCR-RFLP, which suggests that the allelic discrimination rRT-PCR is more sensitive than RT-PCR-RFLP. The results were confirmed by sensitivity evaluation of the assay (Figure 2). Our allelic discrimination rRT-PCR method is different from the loop-mediated isothermal amplification assay used in Japan,[12] because the detection limits (10 copies/μL) of the allelic discrimination rRT-PCR are similar between vaccine and wild-type MeVs, which could help eliminate false-negative results when detecting wild-type MeV infection. Subsequent sequencing and genotyping analysis confirmed that Cases 1 and 8 were caused by the measles vaccine, as sequences generated from the two cases were identical to the Chinese vaccine strain Shanghai-191 (Figure 3).

After confirming the results, we summarized the clinical features of the eight cases by retrospective analysis. Previous studies found that the incubation period for typical measles infection is 10 days to the onset of fever and 14 days to the onset of rash,[25] but this may be prolonged to 37 days post-immunization for vaccine-associated measles.[10] However, the incubation period of the vaccine-associated measles cases identified in our study was probably similar to that of typical measles. The clinical symptoms of 3C were included in the definition of wild-type measles by WHO and were suggested as key positive findings to distinguish wild-type and vaccine MeV infection.[22] Cases 1 and 8 in our study had no 3C symptoms, and the other four cases caused by wild-type MeV presented 3C symptoms randomly. However, vaccine-associated cases reported in Canada documented that the patients developed 3C symptoms, in addition to fever and rash.[10] Significantly, in Case 1, physicians observed Koplik's spots on the patient's oral mucosa, and epidemiologists considered the patient had the probable route of exposure since the patient had been to hospital before onset of illness. Hence, it is unreliable to discriminate between vaccine and wild-type MeV infection only on the basis of clinical symptoms or epidemiological history. In addition, Cases 2—4 and 6 were monitored in Shaoxing City during late March 2013 and early April 2013. During that period, many measles cases were reported in the city. Based on our results, it is reasonable to assume that these patients had already incubated wild-type MeVs before vaccine inoculation or during the period from vaccination to onset of illness.

In this study, not every genotype of MeV was real tested by the developed allelic discrimination rRT-PCR assay since only three genotypes were available in our laboratory. Even though the multiple sequence alignment assures the accuracy of the assay theoretically, we cannot be completely certain what the performance of the allelic discrimination rRT-PCR assay would be in detection of the other 19 genotypes of MeV. Another limitation of the study was that only a small number of specimens (8 suspected cases) were detected using the allelic discrimination rRT-PCR, which may have been insufficient to precisely evaluate the sensitivity and specificity of the assay. Therefore, the assay should be further validated with different genotypes of MeV as well as a large number of clinical samples.

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In conclusion, the process described in this study for the identification and confirmation of vaccine-associated measles cases could improve the accuracy of the measles case surveillance system. Moreover, the allelic discrimination rRT-PCR assay provides a good alternative for differentiating between vaccine and wild-type MeV.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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References

1. Enders JF, Peebles TC. Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc Soc Exp Biol Med* 1954;**86**:277—86.
2. Baker JP. The first measles vaccine. *Pediatrics* 2011;**128**:435—7.
3. Perry RT, Gacic-Dobo M, Dabbagh A, Mulders MN, Strebel PM, Okwo-Bele JM, et al. Progress toward regional measles elimination — worldwide, 2000—2013. *MMWR Morb Mortal Wkly Rep* 2014;**63**:1034—8.
4. World Health Organization. Measles vaccines. *Wkly Epidemiol Rec* 2009;**84**:349—60.
5. Makino S. Development and characteristics of live AIK-C measles virus vaccine: a brief report. *Rev Infect Dis* 1983;**5**:504—5.
6. Strebel P, Papania M, Halsey N. Measles vaccine. In: *Vaccine*. Atlanta: Elsevier; 2004. p. 389—440.
7. Nestibo L, Lee BE, Fonseca K, Beirnes J, Johnson MM, Sikora CA. Differentiating the wild from the attenuated during a measles outbreak. *Paediatr Child Health* 2012;**17**:e32—3.
8. Aoki Y, Mizuta K, Ikeda T, Abiko C, Itagaki T, Ahiko T. Isolation of vaccine-derived measles viruses from children with acute respiratory infection. *Tohoku J Exp Med* 2013;**230**:111—5.
9. Hau M, Schwartz KL, Frenette C, Mogck I, Gubbay JB, Severini A, et al. Local public health response to vaccine-associated measles: case report. *BMC Public Health* 2013;**13**:269.
10. Murti M, Krajden M, Petric M, Hiebert J, Hemming F, Hefford B, et al. Case of vaccine-associated measles five weeks post-immunisation, British Columbia, Canada, October 2013. *Euro Surveill* 2013;**18**. http://dx.doi.org/10.2807/1560-7917.ES2013.18.49.20649.
11. Berggren KL, Tharp M, Boyer KM. Vaccine-associated "wild-type" measles. *Pediatr Dermatol* 2005;**22**:130—2.
12. Nakayama T, Sawada A, Kubo H, Kaida A, Tanaka T, Shigemoto N, et al. Simple method for differentiating measles vaccine from wild-type strains using loop-mediated isothermal amplification. *Microbiol Immunol* 2013;**57**:246—51.
13. Zhou JH, Wang S, Chen C. The establishment of method for identifying China vaccine strains and wild strains of measles virus. *Zhongguo Yi Miao He Mian Yi* 2009;**15**:310—5.
14. Yu BB, Feng Y, Xu CP, Lu YY, Qian J. Simultaneous detection of measles and rubella virus by multiplex real-time RT-PCR with an internal control. *Bing Du Xue Bao* 2010;**26**:109—14.
15. Keniscope C, Juliana R, Subri H, Shangari SR, Wan Nor Azlina WA, Hamizah A, et al. Isolation of measles virus from clinical specimens using B95a and Vero/hSLAM cell-lines. *Med J Malaysia* 2009;**64**:37—40.
16. Bankamp B, Byrd-Leotis LA, Lopareva EN, Woo GK, Liu C, Jee Y, et al. Improving molecular tools for global surveillance of measles virus. *J Clin Virol* 2013;**58**:176—82.
17. Sun Y, Liang M, Qu J, Jin C, Zhang Q, Li J, et al. Early diagnosis of novel SFTS bunyavirus infection by quantitative real-time RT-PCR assay. *J Clin Virol* 2012;**53**:48—53.
18. World Health Orgainization. Update of the nomenclature for describing the genetic characteristics of wild-type measles viruses: new genotypes and reference strains. *Wkly Epidemiol Rec* 2003;**78**:229—32.
19. Tamura KPD, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;**28**:2731—9.
20. Gao J, Chen E, Wang Z, Shen J, He H, Ma H, et al. Epidemic of measles following the nationwide mass immunization campaign. *BMC Infect Dis* 2013;**13**:139.
21. World Health Organization. Manual for the laboratory diagnosis of measles and rubella infection. Geneva: WHO 2007: Available at:http://www.who.int/ihr/elibrary/manual_diagn_lab_mea_rub_en.pdf. [accessed 26.12.14].
22. Choe YJ, Eom HS, Bae GR. Vaccine-associated measles in the low-incidence country of Korea over a 10-year period. *Jpn J Infect Dis* 2014;**67**:180—3.
23. World Health Organization Regional Office for the Western Pacific. 23rd session of Regional Committee. Hanoi, Viet Nam, 2012: Available at:http://www.wpro.who.int/about/regional_committee/63/documents/RC_07_Item_12_Measles_elimination_FINAL_COMPLETE.pdf. [accessed 26.12.14].
24. Helfand RF, Heath JL, Anderson LJ, Maes EF, Guris D,Bellini WJ. Diagnosis of measles with an IgM capture EIA: the optimal timing of specimen collection after rash onset. *J Infect Dis* 1997;**175**:195—9.
25. Moss WJ, Griffin DE. Measles. *Lancet* 2012;**379**:153—64.